Frequent Abnormalities of the P15 and P16 Genes in Mycosis Fungoides and Sezary Syndrome

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There are few data on the molecular pathogenesis of cutaneous T cell lymphomas. A recent allelotyping study by our group identified frequent allelic loss on 9p, 10q, and 17p including losses on 9p21 in 16% of patients with mycosis fungoides and 46% with Sezary syndrome. The P15 and P16 genes are intricately linked on 9p21 and can be inactivated in melanoma and non-Hodgkin's lymphoma. We have therefore studied 76 patients with either mycosis fungoides or Sezary syndrome for abnormalities of these genes. DNA samples were analyzed for loss of heterozygosity, homozygous deletion, intragenic mutations, and promoter methylation. In addition P15 and P16 protein expression was assessed. Microsatellite analysis was informative in 73 of 76 cases: allelic loss on 9p21 was identified in 18 patients (25%), including 12 of 57 with mycosis fungoides (21%) and six of 16 with Sezary syndrome (37%). Single strand conformation polymorphism analysis of the entire coding regions of both genes did not identify any mutations, although two polymorphisms were identified including C613A, which has not previously been described. P15 and P16 gene promoter methylation was found in 45% and 29% of patients, respectively. Furthermore aberrant P15 protein expression was detected in 85% of patients analyzed with P15 gene abnormalities and abnormal P16 expression in 59% with P16 gene abnormalities. These abnormalities were not dependent on cutaneous stage of disease. This study suggests that abnormalities of the P15 and P16 genes are common in both early and advanced stages of mycosis fungoides and Sezary syndrome and that these genes may be inactivated by allelic loss and aberrant promoter methylation. ***Key words: chromosome 9/cutaneous T cell lymphoma/P15 and P16 genes. J Invest Dermatol 118:493–499, 2002***

Primary cutaneous T cell lymphomas (CTCL) consist of mycosis fungoides, Sezary syndrome, and related variants (Edelson, 1980). Mycosis fungoides presents with cutaneous patches and plaques that may progress to tumor stage disease or erythroderma (Willemze *et al*, 1997; Zackheim *et al*, 1999) whereas Sezary syndrome is characterized by erythroderma, peripheral lymphadenopathy, more than 5% circulating atypical lymphocytes (Sezary cells), and a peripheral blood T cell clone as demonstrated by T cell receptor (TCR) gene analysis (Russell-Jones and Whittaker, 1999).

The molecular pathogenesis of both mycosis fungoides and Sezary syndrome is still poorly understood. Previous cytogenetic studies have not identified any characteristic translocations in CTCL although nonrandom clonal chromosomal abnormalities have been detected, many of which involve complex structural rearrangements of multiple chromosomes. A study of 11 patients with CTCL using comparative genomic hybridization found chromosomal losses were more frequent than gains. Abnormal patterns were identified in six patients with frequent losses on chromosomes 10, 13, and 17. Three patients had abnormalities involving chromosome 9, one of which involved a deletion in the region of 9p21 (Karenko *et al*, 1999). Rearrangements involving 9p were also identified in three of six patients with CTCL (Berger *et al*, 1988) and more recently loss of heterozygosity (LOH) at 9p21 was detected in seven of 11 cases of primary cutaneous CD30-positive large cell anaplastic lymphoma with loss of p16 protein in two of these (Boni *et al*, 2000). Furthermore a recent allelotyping study in CTCL by our group identified frequent allelic loss on 9p, 10q, and 17p including losses on 9p21 in 16% of patients with mycosis fungoides and 46% with Sezary syndrome (Scarisbrick *et al*, 2001). These findings suggest that 9p21 harbours one or more tumor suppressor genes that are important in the pathogenesis of CTCL and P15 and P16 are likely candidate genes.

The P16 gene encodes an 18.1 kDa nuclear protein that blocks cell cycle progression at the G1/S transition by binding to cyclin-dependent kinase 4 (CDK4) and CDK6 thereby preventing their ability to phosphorylate the retinoblastoma protein (Serrano *et al*, 1993). Loss of function is associated with loss of this inhibitory effect and could potentially lead to uncontrolled cell growth (Serrano *et al*, 1993; Kamb *et al*, 1994). The P15 gene is located 25 kb centromeric to the P16 gene and encodes a nuclear protein (p15) that has structural homology to p16 and also has CDK4 and CDK6 inhibitor activity. Initial studies found that P15 and P16 gene inactivation was frequently due to homozygous deletion and intragenic mutations were rarely identified (Ogawa *et al*, 1994; Cairns *et al*, 1995). Furthermore several studies identified LOH

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Abbreviations: CDK, cyclin-dependent kinase; LOH, loss of heterozygosity; SSCP, single strand conformation polymorphism.

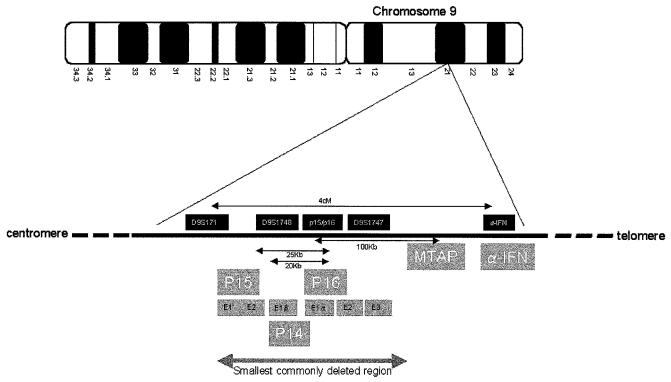

Figure 1. Diagram showing approximate positions of microsatellite markers in relation to the P15 and P16 genes on 9p21.

involving deletion of one or both genes with no detectable mutation on the remaining allele (Ogawa *et al*, 1994; Takeuchi *et al*, 1995). This observation raised questions about the mechanism of gene inactivation and it was suggested that either an epigenetic mechanism produced inactivation of the remaining allele or that a gene dosage effect might be critically important for P15 and P16 gene function, such that loss of one allele could exert a dominant negative effect. Aberrant methylation of CpG islands within P15 and P16 gene promoter sites producing transcriptional silencing was subsequently identified as an alternative mechanism of P15 and P16 gene inactivation in sporadic malignancies (Gonzalez-Zulueta *et al*, 1995; Herman *et al*, 1995; Merlo *et al*, 1995).

Germline mutations of P15 and P16 genes are found in some cases of familial melanoma (Hussussian *et al*, 1994) and homozygous deletions have been found in various sporadic malignancies including lymphoma (Gombart *et al*, 1995; Otsuki *et al*, 1995; Heyman *et al*, 1996) and specifically more aggressive forms of nodal non-Hodgkin's lymphoma (Pinyol *et al*, 1997, 1998; Elenitoba-Johnson *et al*, 1998; Villuendas *et al*, 1998). These findings have led us to perform a comprehensive study of abnormalities of the P15 and P16 genes to determine if gene inactivation is important in the pathogenesis of CTCL. Samples were analyzed for LOH, homozygous deletions, intragenic mutations, and aberrant promoter methylation. In addition P15 and P16 protein expression was studied.

MATERIALS AND METHODS

Sample selection Seventy-six patients with primary CTCL were selected for this study, consisting of 58 mycosis fungoides patients with different cutaneous stages of disease: T1, 14; T2, 18; T3, 20; and T4, 6; this included IA, 14; IB, 15; IIA, 3; IIB, 14; III, 2; and IVA, 10 (Lamberg *et al*, 1984). All patients had skin lesions consistent with a clinical diagnosis of mycosis fungoides, compatible skin histology, and a T cell clone in lesional skin demonstrated by TCR gene analysis. These tumor samples were chosen because either southern blot or single strand conformational polymorphism polymerase chain reaction (PCR) based TCR gene analysis (Whittaker *et al*, 1991; Fraser-Andrews *et al*, 2000) had previously demonstrated a clonal population in lesional skin, which was absent from the peripheral blood or lymph node. Furthermore only those samples with a large dominant clone were selected for this study: a large dominant clone was determined by measuring the signal intensity of the discrete band, using an Image Master Scanning Densitometer (Amersham Pharmacia, U.K.) with Image Master Software (Amersham Pharmacia), and only samples with a signal intensity of more than 50% in the discrete band compared to the background smear were considered to contain a large dominant clone. Eighteen patients with Sezary syndrome were also selected and the diagnostic criteria included erythroderma, peripheral lymphadenopathy, the presence of circulating Sezary cells, and a large dominant peripheral blood T cell clone.

Microsatellite analysis requires comparison between tumor and normal DNA from individual patients. In most patients with mycosis fungoides tumor DNA was extracted from lesional skin and normal control DNA from either peripheral blood lymphocytes or lymph nodes. DNA extraction was performed according to standard procedures by proteinase K digestion (United States Biochemicals) and phenol–chloroform extraction (United States Biochemicals).

In most patients with Sezary syndrome (15 of 18) and in three with tumor stage mycosis fungoides T cell clones were detected in skin, blood, lymph nodes, and bone marrow aspirates. In these patients no DNA was available without a T cell clone and normal DNA was obtained by isolating CD8-positive cells from peripheral blood lymphocytes as previously described (Scarisbrick *et al*, 2001).

LOH analysis Tumor and normal DNA samples from individual patients were analyzed for allelic loss using oligonucleotide primers for several microsatellite markers. Markers were chosen flanking the P15 and P16 genes. These genes are intricately linked on 9p21 and a further marker in between these genes was also selected for analysis. This included primers (from telomere to centromere) D9S171, p15/p16, and α-interferon (α-IFN) (Heyman *et al*, 1996). **Figure 1** shows the relative position of these markers to the P14, P15, P16, methylthioadenosine (MTAP), and the IFN cluster genes on 9p21. DNA samples and matched normal controls from individual patients were analyzed for LOH as previously described (Scarisbrick *et al*, 2000, 2001).

Determination of LOH All samples showing two distinct allelic bands in normal DNA were considered to be informative. Samples that produced a single allelic band in normal DNA or failed to amplify for a given microsatellite marker were scored as noninformative.

The signal intensities for all informative samples were examined visually by two independent observers without knowledge of clinical details. LOH was scored as positive when a clear reduction in signal intensity was detected in one of the alleles in tumor DNA compared to the same allele in the paired normal DNA. In instances where more than one band was present within each allele the exact position of each allele was decided by comparing the banding pattern in all samples analyzed with the same marker and selecting the most consistent pattern: the two alleles were identified as two groups of bands of similar number and signal intensity (Scarisbrick *et al*, 2001). In order to quantify signal intensities we also analyzed samples using a scanning densitometer with Image Quant software (Amersham Pharmacia); a reduction in signal intensity of more than 50% in one of the alleles in the tumor DNA compared to the paired normal DNA was defined as LOH. Many samples showed a reduction in signal intensity approaching 90%. Densitometry was undertaken on all samples where the reduction in signal intensity was difficult to quantify visually. Samples showing LOH were subjected to repeat amplification and analysis for confirmation when sufficient material was available.

Analysis for homozygous deletions Samples with LOH at microsatellite markers flanking the P15 and P16 gene region (p15/p16 and α-IFN for the P16 gene and p15/p16 and D9S171 for the P15 gene) were examined for homozygous deletion using the P16 intragenic marker D9S1747 and the P15 intragenic marker D9S1748. PCR were performed as for LOH analysis. Twenty-five cycles were performed annealing at 55°C–60°C depending on the T_m of the primer pairs. Radiographs were then analyzed for LOH. Any sample with LOH at both markers flanking either P15 or P16 genes, which demonstrated retention of heterozygosity at the intragenic microsatellite marker (due to the presence of wild-type reactive cells within biopsy material), was considered to have homozygous deletion of the appropriate gene.

Single strand conformation polymorphism (SSCP) analysis Samples demonstrating allelic loss on 9p were also analyzed for P15 and P16 gene mutations. Five exonic primer pairs spanning all three exons of the P16 gene and both exons of the P15 gene (Heyman *et al*, 1996) were amplified by PCR prior to SSCP analysis on nondenaturing polyacrylamide gels.

The PCR was performed as for LOH analysis. 6% dimethyl sulfoxide (BDH Laboratories, U.K.) was added for the primer pairs of exon 1 of the P16 gene. Thirty cycles were performed annealing at 55°C–65°C depending on the T_m of the primer pairs.

PCR products were diluted 2-fold with stop solution [95% formamide (BDH Laboratories), 20 mM ethylenediamine tetraacetic acid (BDH Laboratories), 0.05% xylene cyanol FF (BDH Laboratories), and 0.05% bromophenol blue (Sigma, U.K.)] and denatured for 10 min at 95°C prior to rapid cooling in a dry ice ethanol bath. Samples were loaded in turn onto nondenaturing 6% polyacrylamide gels (Gibco BRL, U.K.) containing 0%, 5%, and 10% glycerol (Sigma) and electrophoresed using an S2 sequencing gel apparatus (Gibco BRL) at 4 W overnight.

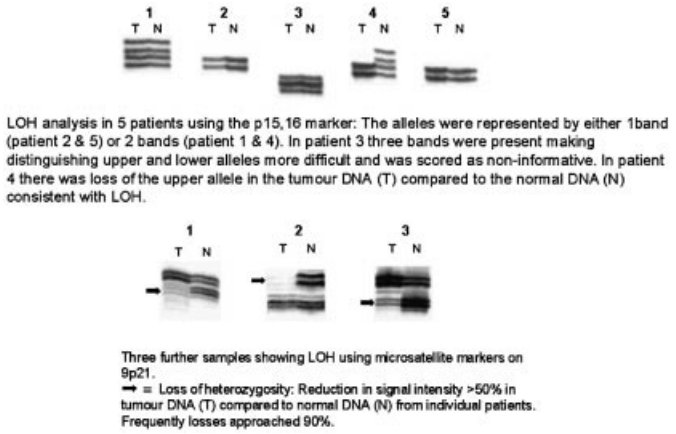

Figure 2. Examples of radiographs showing loss of heterozygosity on 9p21. Microsatellite analysis using different markers produces different allelic banding patterns. LOH needs to be assessed in comparison with other samples using the same marker.

Polyacrylamide gels were dried and exposed to autoradiography (Genetic Research Instrumentation, U.K.). Radiographs were examined for conformational change as shown by a band shift in tumor DNA compared to wild-type.

Sequence analysis Abnormal band shifts detected by SSCP-PCR were sequenced. Each abnormal band shift was cut directly from the dried gel. DNA was recovered by boiling the gel with water. DNA eluted in this way was re-amplified using a 5′ biotinylated primer and the corresponding unmodified 3′ primer. Successful reamplification was determined by electrophoresis on 2% agarose gels prior to sequencing. The DNA strands were then separated using a magnetic rack (Dynal, Norway), which immobilizes the biotinylated 5′ DNA strand allowing the removal of the 3′ DNA strand. The single stranded 5′ DNA was then sequenced using a Sequenase Version 2 DNA sequencing kit (United States Biochemicals, U.K.) with S35 (Amersham Pharmacia). Products were then analyzed by electrophoresis on denaturing 6% polyacrylamide gels with 7 M urea (United States Biochemicals, U.S.A.).

Methylation analysis of CpG islands Samples were analyzed for methylation of the 5′-CpG islands within exon 1 of the P15 and P16 genes, which contain the promoter regions, using methylation-specific PCR (Herman *et al*, 1995). This technique requires DNA to be chemically modified by sodium bisulfite prior to PCR with primers for both methylated and unmethylated products. Methylated cytosine bases are protected from bisulfate conversion to uracil and therefore do not amplify with primers for unmethylated DNA (Heyman *et al*, 1996). RAJI cell line (European Tissue Culture Center, U.K.) was grown in RPMI 1640 (Life Technologies, U.K.) with 10% heat-inactivated fetal bovine serum (Helena Technologies, U.K.) supplemented with 2 mM glutamine (Life Technologies). Cells were harvested by trypsinization and DNA was extracted, as previously described, during the logarithmic growth phase. The RAJI cell line is hypermethylated at the P15 and P16 gene promoter and was therefore used as a positive control.

DNA samples were subjected to bisulfite modification as previously described (Herman *et al*, 1995). Methylation-specific PCR was then performed incorporating radioactivity (P33) using two primer pairs for unmethylated DNA (P16U and P15U) and three primer pairs for methylated DNA, two for methylation of the first exon of P16 (P16M and P16M2) and one for methylation of the first exon of the P15 gene (P15M). PCR products were electrophoresed on denaturing 6% polyacrylamide gels for 2–3 h at 35 W. Autoradiographs were examined for PCR amplification products with both methylated and unmethylated primers for each sample. Any sample that amplified with the unmethylated primers was considered to have undergone bisulfite modification and was assumed to be informative (the presence of wild-type DNA from reactive cells presumably accounting for nonmethylated DNA in biopsy material). On each gel using the methylated primers the RAJI PCR product and at least one informative sample was sequenced to ensure it was the correct product. All informative samples that amplified with the methylated primers for either the P15 or P16 gene were scored as hypermethylated for the appropriate gene.

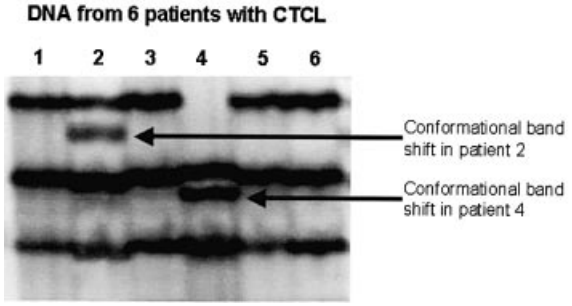

Figure 3. Conformational band shifts in exon 3 of the P16 gene. SSCP gel showing abnormal bands in two patients.

Immunohistochemical methods Immunohistochemical staining was performed manually on freshly cut 4 μm sections of formalin-fixed paraffin-embedded tissue on lesional skin from the same biopsy that DNA was extracted for this study. In brief, sections were melted and dewaxed and endogenous peroxidase was blocked by incubation with 3% hydrogen peroxide (Sigma) in methanol (Sigma) at room temperature. Antigen retrieval was performed by heat-mediated pretreatment using 10 mmol per l sodium citrate buffer (Sigma) at pH 6.0 and microwaved at 650–750 W for 10–15 min. A streptavadin–biotin labeled horseradish peroxidase procedure was then employed in all cases.

Immunohistochemical staining using primary mouse monoclonal antibodies against p15 and p16 (Santa Cruz Technology, CA) at a dilution of 1:250 was performed as previously described (Elenitoba-Johnson *et al*, 1998). An irrelevant antibody of the same subclass was used as a negative control. Sufficient archival material was available for analysis from 20 of 43 patients with P15 gene abnormalities and 17 of 34 patients with P16 gene abnormalities. In addition 10 samples without any detectable gene abnormalities were also analyzed,

Slides were assessed by two independent observers without knowledge of clinical details or molecular studies. p15 and p16 are nuclear proteins and nuclear reactivity with the antibody was considered as a positive signal for protein expression. Inactivation of P15 and P16 genes have been shown to be associated with absent protein expression (Reed *et al*, 1996; Pinyol *et al*, 1998; Villuendas *et al*, 1998). The staining of basal and suprabasal keratinocytes was used as an internal control. The percentage of mononuclear cells showing absent nuclear reactivity was assessed as follows: <25%, 25%–50%, 50%–75% or >75%.

RESULTS

LOH Seventy-three of the samples were informative for at least one of the three microsatellite markers. LOH was observed in 18 samples (25%) at one or more of the markers. In 16 samples this included LOH with the p15/p16 microsatellite marker and in the other two samples there was loss with the α-IFN microsatellite marker and retention of heterozygosity with the p15/p16 marker. The degree of reduction in allelic signal intensity was frequently greater than 50% and in some instances approached 100%. Examples of allelic loss are shown in **Fig 2**.

Twelve of the 58 patients with mycosis fungoides (21%) demonstrated LOH. This included seven of 32 patients (22%) with early cutaneous stages of mycosis fungoides (two of 14 with T1 and five of 18 with T2 stage) and five of 25 patients (20%) with advanced cutaneous disease (four of 20 with T3 and one of five with T4 stage). LOH was also identified in six of 16 patients with Sezary syndrome (38%).

These microsatellite markers on 9p were specifically chosen because of their proximity to the P15 and P16 tumor suppressor genes (9p21) and LOH at these markers suggests that one allele of either gene has been deleted (Heyman *et al*, 1996).

Homozygous deletion Two patients demonstrated LOH in both markers flanking the P16 gene (p15/p16 and α-IFN) and were analyzed for homozygous deletions using the intragenic microsatellite marker D9S1747. Homozygous deletion of the P16 gene was not identified in either sample using this approach. LOH at both markers flanking the P15 gene were not found in any

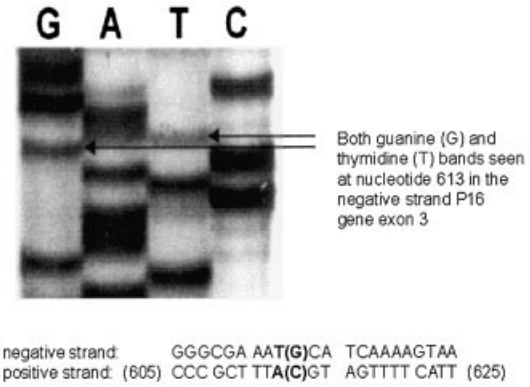

Figure 4. Cytosine (C) to adenosine (A) substitution at nucleotide 613 of the P16 gene. Polyacrylamide gel showing sequencing of the negative strand of P16 exon 3; both guanine (G) and thymidine (T) bands are present with a reduced signal intensity at 613 indicating that the sample is heterozygous for this C(613)A polymorphism.

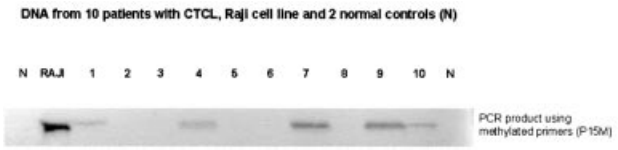

Figure 5. Aberrant methylation of the P15 gene. Polyacrylamide gel showing amplification of DNA extracted from the RAJI cell line with primers for methylated P15 gene promoter in five of 10 patients with CTCL (patients 1, 4, 7, 9, and 10).

patient sample and so we were unable to look for homozygous deletions of the P15 gene using this method.

Mutational analysis of the P15 and P16 genes All 18 patient samples showing LOH on 9p21 were screened for point mutations, small deletions, and insertions in exons 1 and 2 of the P15 gene and exons 1, 2, and 3 of the P16 gene using SSCP-PCR. No abnormal band shifts were detected in either exon of the P15 gene. Three conformational band shifts were detected in exon 3 of the P16 gene (**Fig 3**). Sufficient material for sequence analysis was available in two of these patients and both showed a single base pair substitution residing in the 3′ untranslated region of the P16 gene, which was present in both tumor and normal DNA, consistent with a polymorphism; one represented a previously described polymorphism, C to T base transition at nucleotide 580 (Holland *et al*, 1995), and the other a C to A base transversion at nucleotide 613 (**Fig 4**), which to our knowledge has not previously been reported (http://www.ncbi.nlm.gov/SNP). Both SSCP analysis and direct sequencing demonstrated residual normal bands representing the normal allele suggesting that both patients were heterozygous for these polymorphisms.

In order to determine the frequency of these polymorphisms in our cohort of patients the remaining 58 patients were subjected to SSCP-PCR analysis of exon 3 of the P16 gene. Abnormal band shifts were detected in a further five patients. Sequence analysis was performed and three further patients demonstrated the same C to T base change at nucleotide 580. This represents a T allele frequency at nucleotide 580 of at least 2.6% (four of 152 alleles). The C613A polymorphism was not detected in any other patient sample.

Methylation status of P15 and P16 genes DNA modification by bisulfite was complete in 66 patient samples as detected by DNA amplification with the primer set for modified unmethylated DNA. Thirty-six of these samples (55%) also amplified with at least one of the primer sets for methylated DNA. **Figure 5** shows a radiograph demonstrating amplification with the P15M primer in five of 10 patients. Thirty patient samples (45%) amplified with the methylated primers for exon 1 of the P15 gene (P15M) (**Table I**). This included 12 of 30 patients (40%) with early cutaneous stages of mycosis fungoides (eight of 14 with T1 and four of 16 with T2 stage disease), 13 of 24 patients (54%) with advanced cutaneous disease (11 of 19 with T3 and two of five with T4 stage disease), and five of 12 patients with Sezary syndrome.

Nineteen patient samples (29%) amplified with the methylated primers for exon 1 of the P16 gene (**Table I**), all with mycosis fungoides – 10 of 30 patients (33%) with early cutaneous disease (five of 14 with T1 and five of 16 with T2 stage) and seven of 24 patients (29%) with advanced mycosis fungoides (five of 19 with T3 and two of five with T4 stage). Methylation of the P16 gene was not identified in any of the 12 patients with Sezary syndrome.

Immunohistochemical analysis Twenty patients with P15 gene abnormalities were analyzed for p15 protein expression and 17 patients with P16 gene abnormalities for p16 protein expression using immunohistochemical studies with p15 and p16 monoclonal antibodies, respectively. The molecular abnormalities detected in these patients are shown in **Table I**. Ten patients without detectable abnormalities on 9p21 were also studied.

Altered p15 protein expression was found in 17 of 20 patients and altered p16 protein expression in 10 of 17 patients with gene abnormalities (**Table I**). All patient samples analyzed without any detectable gene abnormalities showed normal protein expression.

In all six samples with biallelic gene abnormalities (three with LOH and P15 methylation and three with LOH and P16 methylation) absent nuclear reactivity was detected in 50%–75% of mononuclear cells (**Fig 6**) but no samples had absent protein expression in all mononuclear cells. The remaining patients with abnormal protein expression and monoallelic gene abnormalities showed absent nuclear reactivity in less than 50% of mononuclear cells (**Table I**).

DISCUSSION

Overall, abnormalities on 9p21 were detected in 63% of patients, including abnormalities involving the P15 gene in 57% and of the P16 gene in 45% of patients, with similar rates detected in all cutaneous stages of mycosis fungoides and Sezary syndrome. Biallelic abnormalities of the P15 gene and P16 gene were identified in nine and six patients, respectively. The most common abnormality was promoter methylation, which was identified in 55% of patients. Allelic loss at the loci of the P15 and P16 genes was detected in 25%. Homozygous deletion was not identified in any patient and no intragenic mutations of either gene were detected. Aberrant P15 protein expression correlated with gene abnormalities in 85% of patients and aberrant P16 gene expression in 59% suggesting gene inactivation.

LOH on 9p21 was detected in 21% of patients with mycosis fungoides, at a similar frequency in both early and late disease, and in 38% with Sezary syndrome. These microsatellite markers on 9p21 were specifically chosen because of their proximity to the P15 and P16 genes and LOH at these markers implies that one allele of either gene has been deleted (Heyman *et al*, 1996). Homozygous deletion is a common mechanism of P15 and P16 gene inactivation and has been reported in patients with T cell leukemia (Quesnel *et al*, 1995; Rasool *et al*, 1995; Takeuchi *et al*, 1995) as well as a subset of patients with non-Hodgkin's lymphoma (Freyling *et al*, 1995; Gombart *et al*, 1995; Koduru *et al*, 1995; Drexler, 1998) where it may be associated with high grade disease (Pinyol *et al*, 1997, 1998; Elenitoba-Johnson *et al*, 1998). These genes are often co-deleted in hematologic malignancies, although cytogenetic and molecular studies have found that the P16 gene is more frequently deleted than the P15 gene (Herbert *et al*, 1994; Dreyling *et al*, 1995; Otsuki *et al*, 1995). We analyzed two samples with LOH at markers flanking the P16 gene using an intragenic microsatellite marker. This technique has been shown to accurately detect homozygous deletion of these genes when compared to southern blot and FISH

Table I. Molecular abnormalities on 9p21 in 48 of 76 patients with CTCL[a]

Molecular abnormalities detected in cohort of 76 patients	No. of patients with abnormalities	No. analyzed for p15 expression	Abnormal p15 expression (no. patients &% negative cells)	No. analyzed for p16 expression	Abnormal p16 expression (no. patients & % negative cells)
LOH (only)	10	3	1 (< 25%)	3	1 (< 25%)
LOH + P15M	5	3	3 (1 > 75%, 2 = 50–75%)	1	0
LOH + P16M	3			3	3 (both 50–75%)
LOH + P15M & P16M	0			0	–
P15M only	14	10	9 (5 = 50–75%, 4 = 25–50%)	0	–
P16M only	5			3	3 (all < 25%)
P15M + P16M	11	4	4 (2 > 75%, 2 = 50–75%)	7	3 (all < 25%)
Total	48	20	17	17	10

[a]P15 and P16 gene expression was analyzed in 20 and 17 patients and was abnormal in 17 (85%) and 10 patients (59%), respectively. The percentage of mononuclear cells with absent nuclear staining was assessed as follows: <25%, 25%, –50%, 50%–75%, >75%.

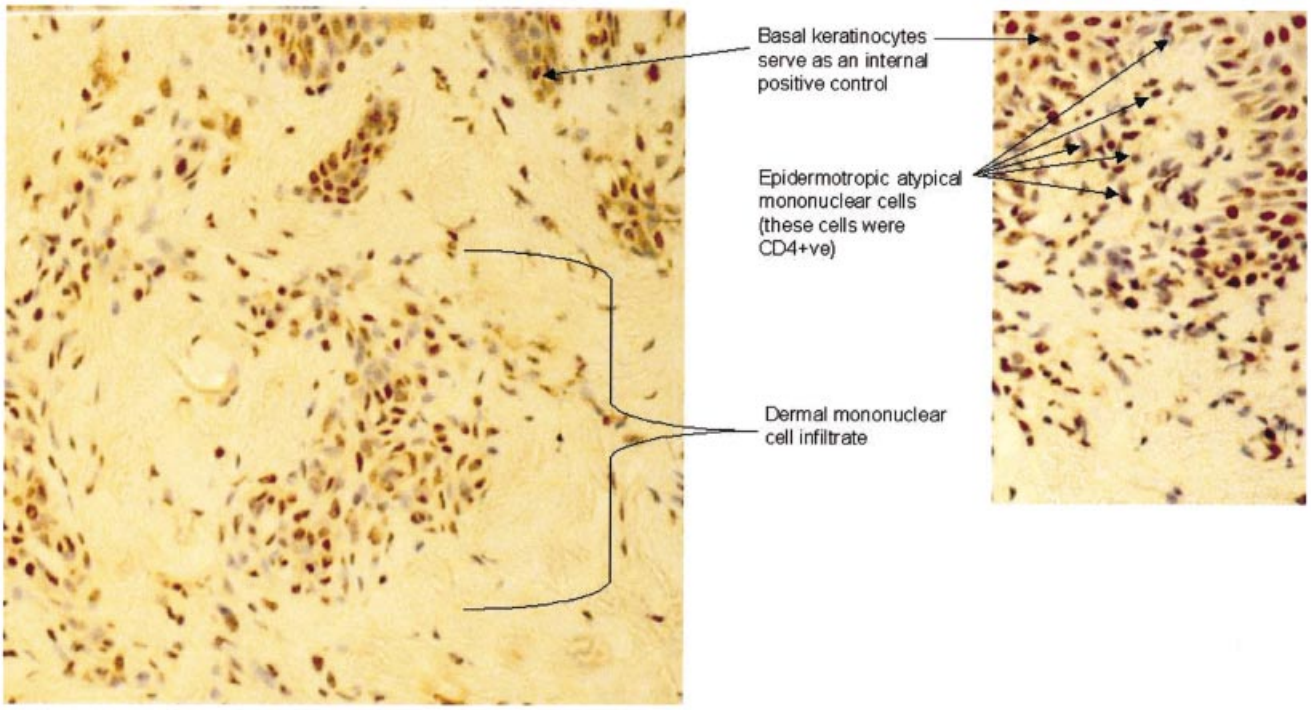

Figure 6. Immunohistochemical staining with a p16 monoclonal antibody in two patients with biallelic P16 gene abnormalities (LOH and p16 gene promoter hypermethylation). The photomicrograph on the left shows normal nuclear staining of the basal keratinocytes (positive internal control) and a dermal mononuclear cell infiltrate with absent staining in 50%–75% of cells; in particular the larger atypical cells are negative. The photomicrograph on the right shows a population of epidermotropic atypical mononuclear cells. These cells are negative for p16.

analysis (Cairns *et al*, 1995) and has previously been used to detect homozygous deletions of the PTEN gene in a subset of patients with mycosis fungoides (Scarisbrick *et al*, 2000). Homozygous deletion of the P16 gene was not detected in either sample using this approach, however. The smallest deleted region on 9p21 commonly involves a 120 kb region from the 3′ MTAP gene to the P15 gene (**Fig 1**) but larger homozygous deletions are common and frequently encompass the entire IFN gene cluster as well as the P15 and P16 genes (Dreyling *et al*, 1995; Olopade *et al*, 1995). Such large homozygous deletions would not be detected using our methodology. Additional methods for detecting larger homozygous deletions such as comparative multiplex PCR or southern blot analysis would determine if the p15 and p16 genes are homozygously deleted in CTCL (Cairns *et al*, 1994; Ogawa *et al*, 1994; Kim *et al*, 1997).

P16 gene mutations are found in a third of patients with familial melanoma as well as in patients with sporadic pancreatic malignancies but have only rarely been identified in nodal lymphomas, in keeping with our findings (Otsuki *et al*, 1995; Gombart *et al*, 1995; Pinyol *et al*, 1997). It is possible that mutations may have been missed as samples were analyzed using SSCP-PCR, which is only capable of detecting those gene mutations that result in an abnormal electrophoretic pattern. Direct sequencing of the entire gene would detect rare single base substitutions not identified by SSCP-PCR but insufficient DNA was available for this approach.

A previously reported P16 gene polymorphism, C580T, was found at a frequency of 2.6% in our cohort, which is lower than the 10% quoted in the literature (Holland *et al*, 1995, 1999). A novel polymorphism (C613A) was detected in one further patient. These single nucleotide substitutions all occurred within the 3′ untrans-

lated region, which is a noncoding region and does not result in an amino acid change. The functional role of the 3′ untranslated region of the P16 gene has still to be determined, however, and may contain sequence elements crucial for gene regulation and expression (Pesole *et al*, 2000).

Several studies have examined the methylation status of the P15 and P16 genes in different forms of lymphoma/leukemia (Herman *et al*, 1997; Martinez-Delgado, 1997; Martinez-Delgado *et al*, 1998; Baur *et al*, 1999). A study of 56 cases of non-Hodgkin's lymphoma, analyzed by both restriction-enzyme-related PCR and methylation-specific PCR, detected high rates of aberrant methylation of P15 and P16 genes in both B and T cell lymphomas particularly in high grade disease (Baur *et al*, 1999). A more recent study of 64 cases of acute leukemia found methylation of the P15 and P16 gene in 40% and 42% of cases, respectively, with low frequencies of mutations and deletions (Guo *et al*, 2000). In our study methylation of the P15 gene was more frequent than of the P16 gene with rates of 45% and 29%, respectively. Methylation of both genes was found in 17% of patients and selective P15 and P16 promoter methylation in 29% and 12% of patients, respectively. In Sezary syndrome selective P15 gene methylation was present in 42% of patients and no cases had alteration of the P16 gene promoter.

A small study by Navas *et al* (2000) of nine patients with patch/plaque and tumor stage mycosis fungoides found alterations in four of nine plaques (hypermethylation in three samples and allelic loss in one) and seven of nine tumors (hypermethylation in five samples and allelic loss in three samples). No point mutations were identified. Peris *et al* (1999) examined mRNA expression in 20 patients with mycosis fungoides by reverse transcription PCR and dot blot hybridization. P16 mRNA expression was undetectable in five patients, intermediate in 13, and normal in two patients. DNA mutational analysis revealed no alterations in the six patients analyzed but methods to detect LOH and hypermethylation were not employed. Absent mRNA expression was found to correlate with absent p16 protein expression, however. No direct correlation between clinicopathologic and molecular findings was evident.

In our study P15 and P16 gene expression was studied in half of the patients with detecteble gene abnormalities and was abnormal in 85% and 59% of patients, respectively, including all six with biallelic gene abnormalities; absent protein expression was found in a subset of the mononuclear cells in these patients with 50%–75% of dermal mononuclear cells showing absent nuclear reactivity. This contrasts with findings in other studies in which P15 and P16 gene abnormalities were associated with absent protein expression in almost all cells (Reed *et al*, 1996; Pinyol *et al*, 1998; Villuendas *et al*, 1998). This difference is presumably explained in mycosis fungoides, however, by the presence of both neoplastic and reactive mononuclear cells within the dermis. Alternatively either P15 or P16 gene promoter hypermethylation may cause a gene dosage effect or gene abnormalities may be restricted to a subpopulation of tumor cells. Fourteen of 17 patients with monoallelic abnormalities of the P15 gene and seven of 14 with monoallelic abnormalities of the P16 gene also showed altered protein expression with absent nuclear reactivity in 25%–50% of mononuclear cells. This suggests that in these patients a subpopulation of tumor cells may have an undetected gene abnormality involving the remaining allele. The other 10 patients with monoallelic gene abnormalities showed normal protein expression implying that in these cases the other allele was functional. Furthermore all 10 patients without detectable gene abnormalities had normal protein expression.

Inactivation of the P16 gene is not specific to lymphoid malignancies and as previously mentioned germline mutations may be found in familial melanoma as well as pancreatic cancer. P16 gene abnormalities have also been identified in other solid malignancies: e.g., in nonsmall cell lung cancer homozygous deletion and hypermethylation have been detected in 31% and 21% of cases with only occasional point mutations and these abnormalites correlated with absent protein expression (Sanchez-Cespedes *et al*, 1999). In oligodendromas homozygous deletion of the P16 gene is frequent but hypermethylation may not be found (Bortolotto *et al*, 2000), whereas in cholangiosarcoma associated with primary sclerosing cholangitis both allelic loss and hypermethylation have been identified and associated with absent protein expression (Ahrendt *et al*, 1999). Several mechanisms for P16 gene inactivation clearly exist; however, no disease-specific pattern of inactivation is apparent.

Further analysis of P15 and P16 gene function is important to determine the transcriptional activity of these genes in different cutaneous stages of mycosis fungoides. Interestingly another tumor suppressor gene on 9p21, the P14 gene, has recently been characterized. This gene has a separate promoter and first exon, designated exon 1β (**Fig 1**), which splices into exon 2 of the P16 gene in an alternative reading frame resulting in a distinct transcript (Quelle *et al*, 1995). The p14 protein can interact with MDM2 protein preventing its degradation and therefore allowing accumulation of p53 (Pomerantz *et al*, 1998; Zhang *et al*, 1998). P14 gene methylation is a common event in colorectal malignancy and is independent of the methylation status of the P16 gene (Esteller *et al*, 2000). Although P14 gene promoter hypermethylation was not detected in 56 cases of non-Hodgkin's lymphoma (Baur *et al*, 1999), deletions on 9p21 would frequently encompass the P14 gene and SSCP analysis of exon 1β and the methylation status of this gene would be appropriate. Insufficient material was available for analysis in this study, however.

P15 and P16 gene abnormalities are frequent in all cutaneous stages of CTCL and commonly involve either allelic loss and/or promoter hypermethylation. In a subset of patients including those with biallelic events p15 and p16 protein expression was abnormal. This suggests that both P15 and P16 gene inactivation may be an early event in the pathogenesis of mycosis fungoides unlike P53 gene inactivation, which is found in advanced disease (Lauritzen *et al*, 1995; Marks *et al*, 1996; McGregor *et al*, 1999).

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